



Case report

Linking the SUMO protease SENP5 to neutrophil differentiation of AML cells



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ABSTRACT

In an mRNA profiling screen performed to unveil novel mechanisms of leukemogenesis, we found that the sentrin-specific protease 5 (SENP5) was significantly repressed in clinical acute myeloid leukemia when compared to healthy neutrophil samples. SENP5 is an enzyme that targets and cleaves small ubiquitin-like modifier (SUMO) residues from SUMOylated proteins. Further investigation with AML neutrophil differentiation cell models showed increased SENP5 expression upon induction of differentiation; in contrast, knocking down SENP5 resulted in significantly attenuated neutrophil differentiation. Our results support a new role of SENP5 in AML pathology, and in particular in the neutrophil differentiation of myeloid leukemic cells.

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1. Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia in adults, with an incidence of 2.5 cases per 100,000 persons worldwide per year [1]. In AML a variety of genetic aberrations, often relevant for cellular differentiation and cell survival, lead to an accumulation of clonal, myeloid precursor cells in the bone marrow and ultimately to failure of hematopoiesis [1]. Acute promyelocytic leukemia (APL) is characterized by the PML-RARA onco-fusion protein that initiates the disease by promoting a block in myeloid differentiation and proliferation of the promyelocytic blasts [2]. APL patients are currently treated with all-trans retinoic acid (ATRA), which directly targets PML-RARA for degradation by activated caspases and the proteasome, thereby overcoming the differentiation block [2]. Interestingly, PML-RARA degradation by arsenic trioxide is triggered by sumoylation [3–5].

In an attempt to identify novel genes with a role in AML differentiation, we performed gene expression profiling experiments focusing on genes involved in cellular proliferation and survival including the sentrin-specific protease 5 (SENP5). SENP proteins are a family of proteins that remove small ubiquitin-like modifiers (SUMO's) from SUMOylated proteins, thereby altering protein function [6]. Recently,

sumoylation was associated with resistance towards chemotherapy in AML [7], but a possible role in differentiation has not been studied yet. We found that SENP5 is downregulated in primary AML patient samples and its expression is induced upon ATRA-mediated neutrophil differentiation of primary APL cells and different AML cell lines. Moreover, impairing SENP5 expression in APL cells decreased ATRA-induced granulocytic differentiation.

2. Materials and methods

2.1. Patient samples

Fresh leukemic blast cells from untreated AML patients at diagnosis were obtained at the Inselspital Bern (Switzerland) and mononuclear cells were separated using a Ficoll gradient (Lymphoprep™, Axon Lab AG, Switzerland). The isolation of primary neutrophils (purity > 95%) was performed by separating blood cells from healthy donors using Polymorphprep™ (Axon Lab AG, Switzerland). CD34⁺ cells from cord blood or bone marrow as well as macrophages were isolated as described [8]. Protocols and the use of human samples acquired in Bern were approved by the Cantonal Ethical Committee.

2.2. Cell lines, differentiation and cell survival

Neutrophil differentiation was performed as described [9]. Successful neutrophil differentiation was assessed by increased CEBPE mRNA

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expression and by surface CD11b expression (#21279114 Immunotools, Friesoythe, Germany) using flow cytometric analysis. Cell viability upon ATRA treatment was measured using an alamarBlue[®] assay (Invitrogen).

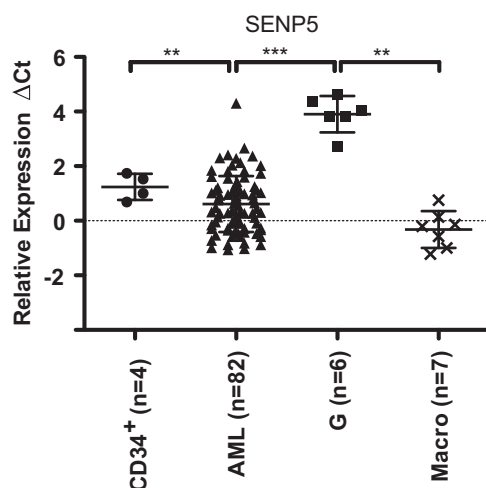


Fig. 1. Inhibition of *SENP5* expression in clinical AML patient samples. *SENP5* mRNA expression was determined in primary AML samples ($n=83$), including FAB M0–M5 AML subtypes, as well as AML with genetic aberrations such as *CEBPA*, *NPM* or *FLT3-ITD* mutations, $t(8;21)$, $t(15;17)$ and $inv(16)$; $CD34^+$ progenitor cells obtained from peripheral blood of multiple myeloma patients ($n=3$) and cord blood ($n=1$); healthy granulocytes ($n=6$) and macrophages ($n=7$). Mann–Whitney– U tests: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.3. Real-time quantitative reverse transcription-PCR (qPCR)

Total RNA was extracted using the RNeasy Mini Kit according to the manufacturer's protocol (Qiagen). Total RNA was reverse transcribed using random primers (Roche Diagnostics) and M-MLV reverse transcriptase (Promega). PCR and fluorescence detection were performed using the ABI PRISM[®] 7700 Sequence Detection System (Applied Biosystems). TaqMan low density arrays were performed and analyzed as described [9]. For quantification of *SENP5* and *CEBPE* the Taqman[®] (Applied Bioscience) Gene Expression Assays Hs00381410_m1 and Hs00357657_m1 were used, respectively. *HMBS* primers and probes have been described [9]. N-fold changes were calculated using the $\Delta\Delta C_t$ method of relative quantification. Data represent the mean \pm s.e.m. of at least triplicate experiments.

2.4. Lentivirus production and transduction

Lentiviral vectors expressing shRNAs targeting *SENP5* were purchased from Sigma-Aldrich (SHCLNG-NM_152699.2-300s1c1 and SHCLNG-NM_152699.2-1325s1c1). Lentivirus production, transduction and selection of cell populations were done as described [9].

2.5. Statistical analysis

Nonparametric Mann–Whitney U tests were applied to compare the difference between 2 groups using the program Prism (Graph-Pad). p values less than 0.05 were considered to be statistically significant.

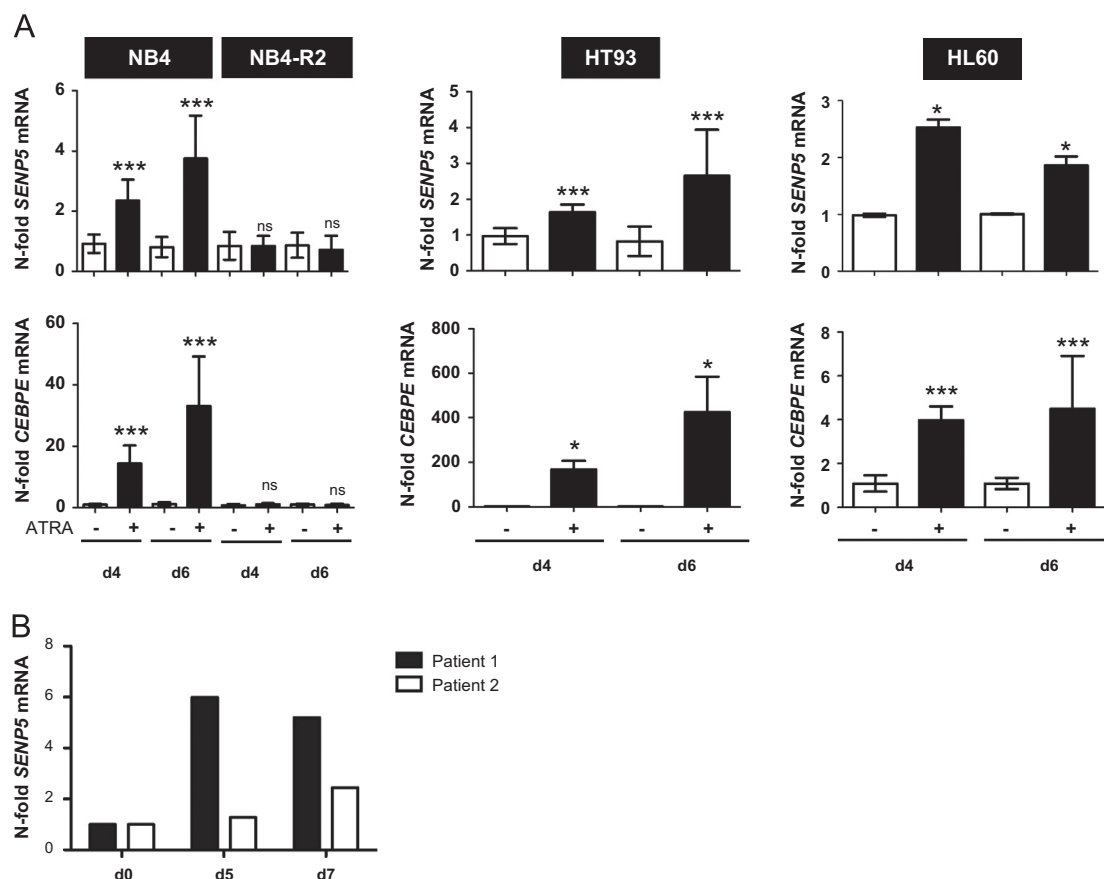


Fig. 2. Induction of *SENP5* expression upon neutrophil differentiation of AML cells. (A, upper panels) *SENP5* qPCR analysis in NB4, NB4-R2, HT93 and HL60 cells. Total mRNA was extracted at day 4 and day 6 after differentiation with 1 μ M ATRA. Values were normalized to the expression of the housekeeping gene *HMBS* and are shown as n-fold mRNA expression to the levels of the control at day 4 of treatment with ATRA. (A, lower panels) *CEBPE* qPCR analysis in NB4, NB4-R2, HT93 and HL60 cells treated and analyzed as in (A). Induction of *SENP5* message in two patients with newly diagnosed APL $t(15;17)$, treated with orally administered ATRA at a dosage of 45 mg/m² daily. Total RNA was extracted from blast cells isolated using a Ficoll gradient and expression levels of *SENP5* were assessed by qPCR. Values were normalized to *HMBS* and day 0 as the experimental starting point. Mann–Whitney– U tests: * $p < 0.05$, *** $p < 0.001$.

3. Results and discussion

3.1. Low *SENP5* expression in primary AML blast cells

We first investigated whether *SENP5* may be involved in AML pathology by quantifying *SENP5* mRNA expression in 82 of 83 primary AML patient samples and comparing expression levels to healthy granulocytes, macrophages or CD34⁺ progenitor cell samples (Fig. 1). The analysis of *SENP5* mRNA levels in granulocytes (mean $\Delta Ct=3.9$) or macrophages (mean $\Delta Ct=-0.32$) from healthy donors, or in CD34⁺ progenitor cells (mean $\Delta Ct=1.24$) showed significantly higher *SENP5* message expression in granulocytes as compared to the other hematopoietic cells ($p<0.01$). Most importantly, the *SENP5* expression in AML cells was downregulated approximately 6-fold (mean $\Delta Ct=0.61$), as compared to

granulocytes ($p<0.001$). These results indicate that higher *SENP5* expression is associated with the granulocyte lineage, whereas immature CD34⁺ progenitors, macrophages, and AML blast cells display lower *SENP5* message levels.

3.2. *SENP5* expression is induced during neutrophil differentiation of AML cells

Intrigued by the low *SENP5* expression in immature AML blast cells as compared to healthy mature neutrophils, we decided to quantify *SENP5* mRNA expression during ATRA induced neutrophil differentiation of three *in vitro* models, namely NB4 and HT93 APL as well as HL60 AML-M2 cells. First, we observed a significant 2.3- and 3.7-fold increase of *SENP5* mRNA expression in NB4 cells at day 4 and 6 of differentiation, respectively. Similarly, HT93 APL

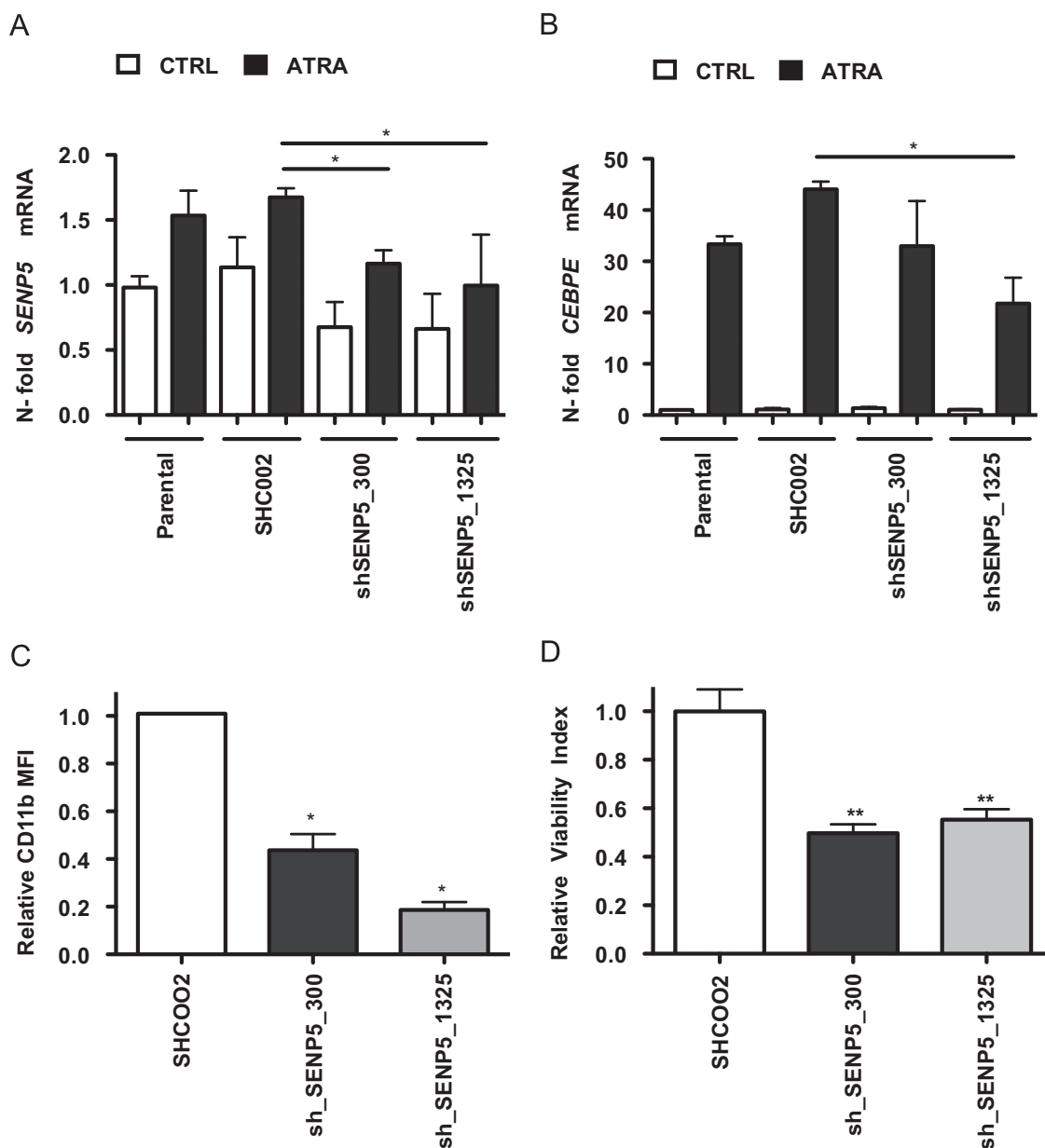


Fig. 3. Knocking down *SENP5* attenuates neutrophil differentiation and survival of NB4 APL cells. (A) NB4 cells were stably transduced with non-targeting shRNA (SHC002) or shRNAs targeting *SENP5* (shSENP5_300 or shSENP5_1325) and differentiated with 1 μ M ATRA for 4 days. *SENP5* mRNA levels were quantified by qPCR and analyzed as in (2A). (B) *CEBPE* qPCR of control and *SENP5* knockdown cells treated as in (A). (C) CD11b flow cytometric analysis of NB4 control (SHC002) and *SENP5* knockdown cells. The values are shown as median fluorescence intensity (MFI) of CD11b expression normalized to the control. (D) alamarBlue cell viability assay of NB4 control and *SENP5* knockdown cells at day 6 of ATRA treatment. Values were normalized to the respective untreated control first, and then to the SHC002 stimulated control. Mann–Whitney–U tests: * $p<0.05$, ** $p<0.01$.

cells displayed a 1.6- and 2.6-fold increase of *SEN5* message at day 4 and 6, respectively. Extending our analysis to HL60 cells, we showed that increased *SEN5* mRNA expression during neutrophil differentiation is not APL specific (Fig. 2A, upper panels). Successful neutrophil differentiation was monitored at day 4 and 6 by CD11b cell surface expression (not shown) and by *CEBPE* mRNA expression with qPCR (Fig. 2A, lower panels). In order to exclude that upregulation of *SEN5* was due to an ATRA-induced unspecific stress response, we repeated the differentiation experiments in ATRA-resistant NB4-R2 cells, which do not respond to ATRA treatment. No significant increase of *SEN5* mRNA expression was observed in these cells (Fig. 2A, left panel). Linking our *in vitro* data to primary patient samples, we found that *SEN5* mRNA levels were increased up to 2.4- and 6-fold in two APL patients receiving oral ATRA and analyzed after short-term follow up (Fig. 2B). Our results clearly point to a role for *SEN5* during neutrophil differentiation of APL as well as non-APL cells.

3.3. Knocking down *SEN5* significantly attenuates neutrophil differentiation and survival of NB4 APL cells

Next, we evaluated whether *SEN5* is functionally involved in neutrophil differentiation of AML cells. Stable NB4 *SEN5* knockdown cell lines were generated using two different lentivirally delivered small hairpin (sh) RNAs targeting *SEN5*. *SEN5* downregulation was confirmed by qPCR (Fig. 3A). The neutrophil differentiation marker *CEBPE* displayed 20–50% decreased expression in the different NB4 *SEN5* knockdown cells upon ATRA-induced differentiation as compared to control cells (Fig. 3B). We next confirmed the effect of *SEN5* inhibition on granulocyte differentiation using a second marker, CD11b: NB4 *SEN5* knockdown cell lines showed a 56% (sh*SEN5*_300) and 81% (sh*SEN5*_1325) decrease in CD11b expression as compared to control cells (Fig. 3C). Interestingly, downregulation of *SEN5* mRNA in AML cell lines also resulted in significantly reduced cell viability upon differentiation (Fig. 3D).

Overall, inhibition of *SEN5* expression in AML cell lines resulted in significantly reduced neutrophil differentiation and cell viability upon ATRA treatment as compared to control cells. To our knowledge, this is the first study to investigate *SEN5* regulation in primary acute myeloid leukemias and primary healthy myeloid cells. First, we found that *SEN5* mRNA expression is significantly downregulated in primary AML as compared to healthy neutrophils. Second, low *SEN5* mRNA levels were associated with an immature myeloid phenotype as evidenced by increased transcript levels of this gene during ATRA-treatment of AML cell lines and APL patients. Importantly, knocking down *SEN5* in an APL neutrophil differentiation model resulted in significantly attenuated granulocyte differentiation and cell viability. The low *SEN5* expression seen in AML cells may promote increased SUMOylation of proteins involved in cellular differentiation and cell survival, thus potentially contributing to the myeloid differentiation block. It has been reported that the nuclear form of *SEN5* co-localizes with the promyelocytic leukemia protein (PML), a key player in leukemogenesis, possibly altering a variety of biological functions [10]. We propose that additional studies may identify the

mechanisms by which this SUMO protease contributes to myeloid differentiation.

4. Authorship and disclosure

EAF, SG and DS designed and performed the experimental research. JJ performed additional *SEN5* knockdown experiments. MFF and BET instigated the experimental design and revised the drafted article. MPT designed the project, wrote the paper and gave final approval of the submitted manuscript. This article is based on the second author's medical doctoral dissertation. All authors approved the final version of the manuscript and have no conflicts of interest to declare.

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